Comparison of three methods for the detection of Epstein-Barr virus in Hodgkin's lymphoma in paraffin-embedded tissues

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Abstract. The percentage rate of Epstein-Barr virus (EBV)-positive cases of Hodgkin's lymphoma (HL) ranges between 20 and 70% in various studies worldwide. To further explore the definite rate in China, three methods, including immunohistochemistry for EBV latent membrane protein 1 (LMP1), *in situ* hybridization (ISH) for EBV-encoded RNA (EBER)-1 and polymerase chain reaction (PCR) for EBV *Bam*HI-W fragment, were employed to detect EBV in 59 cases of HL in China using paraffin-embedded tissue samples. Our results revealed that the PCR method presented the highest (44/59, 74.6%) detection rate among the three methods. The other two methods identified 66.1% (39/59, LMP1) and 67.8% (40/59, EBER1 ISH) EBV-positive results, respectively. Three samples were positive for LMP1 but negative when using EBER1 ISH, while another four samples were EBER1-positive but LMP1-negative. Of the four major histopathological subtypes of HL, the lymphocyte predominant (LR) subtype is the one most frequently associated with EBV, followed by the mixed cellularity (MC), nodular sclerosis (NS) and lymphocyte depletion (LD) subtypes. Our results also indicated the seldomly reported fact that EBV-positive cases in children were more numerous than those of adults with HL.

Introduction

Substantial evidence implicates the Epstein-Barr virus (EBV) in the pathogenesis of Hodgkin's lymphoma (HL) (1-3). EBV detection in HL may be used to risk-stratify patients and derive optimum treatment strategies. Investigation into the presence of EBV nucleic acids in affected tissues in EBV-associated diseases is performed by a variety of different techniques, including spot hybridization, *in situ* hybridization (ISH) and the polymerase chain reaction (PCR) (2,3). EBV-related proteins, including EBV nuclear antigen 1 (EBNA1) and the latent membrane proteins (LMP1, LMP2a and LMP2b) have also been examined by performing immunohistochemical assays. As previously stated, the percentage of EBV-positive cases of HL varied among studies, ranging between 20 and 70% (1), and one of the most significant causes for this wide range may be the sensitivity of the method employed. To obtain an accurate percentage for the EBV infection rate in China, three different EBV detecting methods were employed to analyse 59 paraffin-embedded tissue samples from national cases of HL.

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Materials and methods

Materials and samples. In total, 59 formalin-fixed and paraffin-embedded archival blocks, obtained between 1997 and 2009, were retrieved from the pathology departments of four hospitals: Nanfang Hospital affiliated to the Nanfang Medical University, Guangzhou General Hospital of the People's Liberation Army, Guangzhou Children's Hospital and Shaanxi Provincial People's Hospital. All sections had previously been diagnosed as positive for HL and were re-identified by two of our pathologists. The diagnosis of HL was established by finding H/RS cells within an appropriate background of reactive cells, according to the criteria of the latest WHO classification (4) and also based on morphological (H&E section and immunophenotypic criteria (expression of CD20, CD43 and CD45RO antigen). The study was approved by the ethics committee of Shaanxi Provincial People's Hospital and written informed consent was obtained from the patients.

Immunohistochemistry (IHC). Paraffin sections were stained with MAbs (Dako, Carpinteria, CA, USA) against CD45RO antigen, CD20 antigen (L26), CD45RO antigen (UCHL1), CD15 antigen and CD30 antigen using a standard SP immunohistochemistry kit supplied by Beijing Zhongshan Biological Company (Beijing, China). LMP-1 was detected using a commercial cocktail of MAb against LMP1 (CS1-4, Dako), diluted at 1:200. The IHC procedure was performed as described previously (5). Diaminobenzidine (DAB) was used as a chromogen. Known EBV-positive HL cases were used as positive controls. Each case was tested a minimum of two or three times.

Table I. Comparison of the EBV-positive rate observed using different detection methods in 59 cases of HL.

Type of HL	Cases (%)	Positive cases (%)		
		LMP1	EBER1	PCR (*Bam*HI-W)
LR	30 (50.8)	21 (70.0)	22 (73.3)	24 (80.0)
MC	18 (30.5)	12 (66.7)	13 (72.2)	13 (72.2)
NS	8 (13.6)	4 (50.0)	3 (37.5)	4 (50.0)
LD	3 (5.1)	2 (66.7)	2 (66.7)	3 (100)
Total	59 (100)	39 (66.1)	40 (67.8)	44 (74.6)

EBV, Epstein-Barr virus; LMP1, latent membrane protein 1; EBER1, Epstein-Barr virus early RNA; PCR, polymerase chain reaction; HL, Hodgkin's lymphoma; LR, lymphocyte-rich; MC, mixed cellularity; NS, nodular sclerosis; LD, lymphocyte depletion.

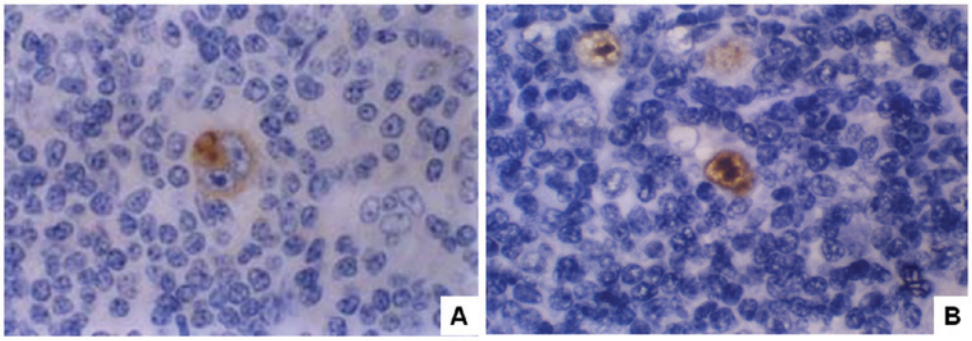

Figure 1. Two cases of Hodgkin's lymphoma in which H/RS cells expressed (A) EBV latent membrane protein 1 (LMP1) and (B) EBV-encoded early RNA1 (EBER1). DAB staining with H&E counterstaining; magnification, x1,000. EBV, Epstein-Barr virus.

EBV-encoded RNA (EBER)1 ISH. EBER expression was detected using 20 bp of doubled digoxigenin-labeled (5' end) oligonucleotide probes (antisense), 5'-ctacagccacacacgtctcc-3', designed by Primer 5.0 software, as the EBER1 gene fragment (GeneBank gil16326314|AB065135.1, human herpesvirus 4 gene for EBER1 small RNA). The probe was synthesized and labeled by Takara Biotech Co. (Dalian, China). The ISH procedure used was described in the protocol of Boster Biotechnology Co. (Wuhan, China).

Briefly, the paraffin sections from each case were mounted on APES-treated glass slides, dewaxed and hydrated, predigested with pepsin (3%) for 5-10 min and hybridized for 14-16 h with a probe concentration of 2 ng/μl. The slides were washed with 2X SSC, 0.5X SSC and 0.2X SSC for 15 min at 37˚C, blocked with BSA at 37˚C for 30 min, treated with biotinylated-rabbit antibodies against digoxin at 37˚C for 60 min and washed with 0.5 M PBS for 5 min four times. SABC was added at 37˚C for 20 min and biotin-peroxidase at 37˚C for 20 min, then the sections were washed with 0.5 M PBS for 5 min four times, dyed with DAB for 10 min and counter-stained with hematoxylin for 8 min. Two known EBV-positive cases were routinely used as positive controls. Two slides treated without the probe were used as negative controls.

PCR techniques

DNA preparation. DNA was extracted from the formalin-fixed paraffin-embedded tissues. Sections (7 μm) were cut from each block and deparaffinized by three changes of xylene followed by ethanol washing. The samples were suspended in 50 μl TE buffer, containing 10 mM Tris-hydrochloric acid (pH 8.0) and 1 mM EDTA (pH 8.0). The DNA was purified using Qiagen columns commercial kit (QIAamp DNA Mini kit; Qiagen, Shanghai, China), and when negative amplification for β-globin (housekeeping gene) was encountered, DNA was re-extracted.

PCR procedure. The first primer pair was a housekeeping gene β-globin: (PC04, 5'-caacttcatccacgttcacc-3'; GH20, 5'-gaagagccaaggacaggtac-3'; expected size 267 bp). The second was designed covering 253 bp of the EBV *Bam*HI-W fragment, based on the DNA sequences of GenBank (forward, 5'-aatgggcgccattttgt-3' and reverse, 5'-tccctagaactgacaatt-3'). The PCR mixture contained 2 μl template DNA, 2.0 μl 10X PCR buffer [containing 100 mM Tris-HCl pH 9.0, 100 mM KCl, 80 mM $(NH_4)_2SO_4$ and 0.1% NP40], 2.0 mm $MgCl_2$, 400 μM dNTP mixture, 10 pmol of each primer and 1.5 units *Taq* Polymerase (Takara Bio, Inc.) in a final volume of 20 μl. The PCR procedure consisted of initial incubation for 5 min at 94˚C, 30 cycles of 94˚C for 30 sec, then 56˚C for 30 sec, 72˚C for 30 sec and a final extension at 72˚C for 5 min. PCR products were visualized under short-wavelength ultraviolet light following ethidium bromide staining of the agarose gels.

Results

Clinical data. There were 59 cases in total, 42 males and 17 females with a gender ratio of 2.5:1. The average age was

Table II. Summaries of clinicopathological findings and EBV results.

Cases	Age/gender	Histological type	LMP1	EBER1	PCR
2	57/F	MC	-	+	+
8	36/M	NS	-	-	+
11	19/M	LR	-	-	+
13	9/M	MC	-	+	+
15	49/M	LR	-	+	+
31	28/F	NS	+	-	+
33	21/M	LR	+	-	+
43	34/M	LR	-	+	+
56	9/F	LD	+	-	+

EBV, Epstein-Barr virus; M, male; F, female; LMP1, latent membrane protein 1; PCR, polymerase chain reaction; EBER1, Epstein-Barr virus-encoded RNA; LR, lymphocyte predominance; MC, mixed cellularity; NS, nodular sclerosis; LD, lymph depletion.

Table III. Comparison of EBER1 and LMP1 between the young and adult HL patients.

Age (years)	LMP1 (%)	EBER1 (%)	Total
<18	26/27 (96.3)[a]	26/27 (96.3)[a]	27
>18	13/32 (40.6)[b]	14/32 (43.8)[b]	32
Total	39/59 (66.1)	40/59 (67.8)	59

[a,b]Significant difference of LMP1 and EBER1 between young and adult groups (P<0.01). EBER1, Epstein-barr virus encoded RNA; LMP1, latent membrane protein 1; HL, Hodgkin's lymphoma.

24.7 (range, 3-57) years old. The number of adolescent patients with HL was 27 (45.8%), with 18 males and 9 females. Out of these cases, 30 were the lymphocyte predominance subtype (LR), 18 cases had mixed cellularity (MC), 8 cases had nodular sclerosis (NS) and 3 cases had the lymphocyte depletion (LD) subtype. These results are summarized in Table I.

LMP1 and EBER1 expression. The LMP1-positive cases demonstrated staining of the membrane and plasma of H/RS cells (Fig. 1A). Of the 59 cases, 39 (66.1%) were shown to be LMP1 positive with the proportions of LR, MC, NS and LD subtypes revealed as 70.0 (21/30), 66.7 (12/18), 50.0 (4/8) and 66.7% (2/3), respectively. By contrast, the H/RS nuclei were dyed using EBER1 ISH as described previously (4,5) (Fig. 1B). Of the 59 cases, 40 (67.8%) were revealed to be EBER1-positive using EBER1 ISH detection (Table I). Notably, among the 18 LMP1-positive cases, 3 weakly LMP1-positive cases (cases 31, 33 and 56) could not be stained in the repeated EBER1 ISH attempts, while another 4 EBER1-positive cases (cases 2, 13, 15 and 43) were LMP1-negative (Table II). Significantly, we found that 26/27 (96.3%) cases of young patients (<18 years old) were LMP1- and EBER1-positive, while, by contrast, only 13/32 (40.6%) adult patients were positive for LMP1 and EBER1 (Table III).

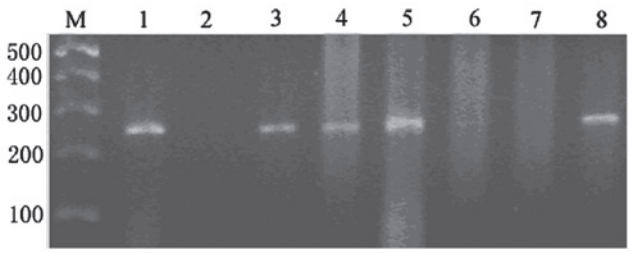

Figure 2. Results of PCR for 253 bp of the EBV *Bam*HI-W fragment. M, marker; lane 1, positive control; lane 2, negative control; lanes 3-8, samples; lanes 3, 4, 5 and 8, positive; lanes 6 and 7, negative. PCR, polymerase chain reaction; EBV, Epstein-Barr virus.

EBV gene expression. Using PCR, 44 of 59 (74.6%) cases were identified as positive for EBV *Bam*HI-W fragment amplification, including 24/30 cases of LR, 13/18 cases of MC, 4/8 cases of NS subtype and 3/3 cases of the LD subtype (Table I and Fig. 2). Seven cases (cases 2, 13, 15, 31, 33, 43 and 56) which were identified as either LMP1- or EBER1-negative, were all recognized as positive using the PCR detection method (Table II).

Discussion

Previous studies have shown that EBV examination may aid in making a correct diagnosis, developing treatment and finding an exact prognosis for EBV-associated diseases (3), thus efficient EBV detection is extremely important. In order to compare the detection rates of the EBV identification methods, three techniques, ISH for EBV early RNA1 (EBER1) sequences, IHC for LMP1 and PCR for EBV *Bam*HI-W fragments, were used to detect the EBV status of 59 HL cases from China using paraffin-embedded tissues.

The results revealed that >66% of cases were identified as EBV-positive, which is a much higher percentage than that produced by Huang *et al* from Northern China (39%, EBER ISH method) (6) and Fatima *et al* from Pakistan (60%, EBV-LMP1 method) (4). The reason for this high incidence may mostly be as our cases include more young patients (27 out of 59 cases). Out of our 27 young cases, 26 (96.3%) exhibited positive EBV results using either the LMP1 or EBER detection methods. It has been reported that the frequency of EBV-positive cases in children is extremely high (between 83 and 100%) in developing countries (7). For example, there were 96.6% EBV-positive cases reported in Indian children and 90.3% in Brazilian children (8,9), which we will examine later in this discussion.

Out of the three EBV detection methods employed, we found that the PCR method yields a higher EBV-positive detection rate (74.6%, 44/59) than that of the LMP1 (66.1%, 39/59) or EBER1 (67.8%, 40/59) methods (Table I). We also found two cases that were negative for LMP1 and EBER but that were positive using PCR detection (Table II). The reason for this may be that the target DNA is able to be amplified by thousands of times by the PCR procedure, thus the PCR method may have a higher sensitivity than the other two methods. However, the PCR method was unable to provide definite information concerning the cellular localization of the EBV-positive cells.

As for the other two methods, it appeared that there was no significant difference between the LMP1 (66.1%, 39/59) and EBER ISH (67.8%, 40/59) techniques. Notably, we found that three samples that were LMP1-positive proved to be EBER1-negative, while another four EBER1-positive cases were LMP1-negative (Table II). Repeated experiments revealed the same results. All these cases appeared EBV-positive when using the PCR detection method. Although these two methods were less sensitive than the PCR method, they provided more information about the localization of EBV-positive cells. We therefore recommend that at least two methods (PCR and either the LMP1 or EBER methods) be performed simultaneously to obtain the most accurate results for EBV infection detection.

In our experiment, we found that the majority of EBER1 and LMP1 expression occurs in an all-or-nothing manner in H/RS cells, but that in certain cases only a small section of the focal H/RS cells were EBV-positive. One interpretation of this may be that some cells are destroyed during the sample preparation process (10-13). EBER is RNA that is preserved in paraffin-embedded tissue, which is easy to destroy during tissue preparation. To avoid false-negative rates, we recommend that several factors be considered prior to deciding that LMP1 or EBER expression is negative. Positive and negative controls should be performed during the experiments and all slide fields should be scanned in the diagnosis. For focal H/RS cells to be deemed EBV-positive, EBER and LMP1 detection methods should be simultaneously performed if possible.

EBV association in HL also depends on age, subtype of HL, location and other characteristics of the study population (3). Notably, almost all of our young cases (26/27, 96.3%) were LMP1- and EBER1-positive, which is a much higher percentage than that of African (68%) (14), Brazilian (77%) or Mexican (65%) (15) cases and it is similar to results of Honduran (100%) (16) and Peruvian (100%) (17) cases. Our results support the view that an association of EBV with childhood HL may vary as a function of histological subtype and socio-economic status (18,19). Concerning the HL subtype, most investigators regard the MC subtype as most frequently EBV-associated (70%), followed by LR (50%), NS (20%) and LD (<5%) subtypes (3,19). Our results are slightly different, LR was observed to be the most frequent (73.3%), followed by MC (72.2%), LD (66.7%) and NS (37.5%) according to the results of our EBER detection. This difference may be attributed to the choice of HL cases.

In conclusion, in the present study, we compared three EBV detection methods in 59 cases of HL. The results demonstrated that the PCR method is the most sensitive of the three, but that it is unable to provide definite information with regard to cellular localization of the EBV-positive cells, while the LMP and EBER methods provided such information. We recommend that at least two methods be performed simultaneously to obtain the most accurate results for EBV infection.

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